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Variations of blood lymphocytes during work studied by cell surface markers, DNA synthesis and cytotoxicity

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SUMMARY

Highly purified peripheral blood lymphocytes from healthy individuals were obtained from samples collected before and after a standardized bicycle ergometer test. The physical activity resulted in a marked increase of circulating lymphocytes. The proportion of T lymphocytes estimated as cells forming rosettes with sheep red blood cells after incubation in the cold decreased, whereas a corresponding increase of cells with receptors for C3, IgG-Fc or surface immunoglobulin was noted. Moreover, after work an increase of cells simultaneously reacting with cell surface markers usually designed as T- or B-cell markers occurred. The reactivity of lymphocytes collected after work in response to Con A, PHA, PWM and PPD was impaired, whereas the slight response to LPS was unchanged. The K-cell cytotoxicity of lymphocytes collected after work increased. The data indicate that physical activity leads to the mobilization of lymphocytes from as yet undetermined sites and with changed composition and reactivity.

INTRODUCTION

The basal level of circulating white blood cells in man can be rapidly and substantially increased by physical activity which mobilizes cells normally sequestered in undetermined sites (for references, see Ahlborg, 1967). Short time exercise results in a phase of lymphocytosis which is followed by a neutrophilic phase during prolonged exercise. Physical activity may lead to alteration of lymphocyte subpopulations (Steel, Evans & Smith, 1974) and to subsequent changes of lymphocyte functions. Knowledge of physiological variations of peripheral blood lymphocytes in healthy persons is essential and an important basis for studies of lymphocytes in patients.

In the present investigation peripheral blood lymphocytes from healthy normal persons, obtained before and after exercise on a bicycle ergometer, were characterized with cell surface markers and by DNA synthesis induced by mitogens and antigens. Antibody-dependent cell-mediated cytotoxicity (ADCC), which is a property of unidentified lymphocytic cells, has also been studied (Perlmann, Perlmann & Wigzell, 1972).

MATERIALS AND METHODS

Fifteen healthy male volunteers between 22 and 30 years of age were included in the study. The participants were investigated at 08.00 hours in the fasting state. They were instructed to avoid exercise before the test. A centrally located venous catheter was inserted through a median cubital vein followed by 30 min of rest in supine position. 140 ml of blood was withdrawn through the catheter and defibrinated by rotation in bottles with glass beads. Bicycle ergometer work was performed by stepwise increase of the work-load until a heart rate of 150 beats/min was reached within 5 min. This work-load was then kept for 10 min, after which 60 ml of blood was collected. Samples were simultaneously taken for estimation of total white blood cells, differential counts, haemoglobin concentration and haematocrit.

Preparation of lymphocytes. Defibrinated venous blood was sedimented in gelatine to remove most of the red blood cells, followed by iron treatment to remove phagocytic cells. These procedures give a preparation of $>99\%$ small lymphocytes

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with a viability of >95% cells as revealed by the trypan blue exclusion test (Holm *et al.*, 1975). Lymphocytes were then taken off for DNA synthesis experiments while lymphocytes to be tested by cell surface markers or cytotoxic reactions (see below) were further centrifuged through a Ficoll–Isopaque gradient to remove remaining red blood cells (Böyum, 1968).

Identification of lymphocytes. Lymphocytes rosetting with sheep red blood cells (SRBC) after incubation in the cold (E-binding cells) were determined. Lymphocytes with receptors for complement (C3) were identified as cells forming rosettes with SRBC coated with rabbit anti-SRBC serum and human complement (EAC-binding cells). Fc-receptor lymphocytes were counted as cells binding SRBC coated with the IgG fraction of rabbit anti-SRBC serum (EA-binding cells). Lymphocytes bearing surface immunoglobulins were identified with indirect immunofluorescence using a polyvalent rabbit anti-human Ig serum (Cappel Laboratory, Downingtown, U.S.A.) followed by a sheep anti-rabbit Ig serum conjugated with fluorescein isothiocyanate (State Bacteriological Laboratory, Stockholm, Sweden).

For simultaneous identification of cells carrying surface immunoglobulins and receptors for complement, Fc-receptors or binding SRBC were performed by first staining for immunoglobulins followed by rosetting as described.

All tests were performed in duplicates. The methods have been described in detail previously (Holm *et al.*, 1975).

DNA synthesis. Iron-purified lymphocyte preparations were suspended in RPMI 1640 medium with Hepes buffer supplemented with 2 mM glutamine, 100 i.u. penicillin, 100 μg streptomycin per ml and 15% heat-inactivated human AB serum. 5×10^5 cells in 1·5 ml medium were used per culture tube. The cells were incubated at 37°C in humid air with 5% CO_2 for 72 hr 0·1 μCi [2-^{14}C]thymidine (specific activity 60 μCi/mM) (Radiochemical Centre, Amersham, Bucks.) per tube was added during the last 24 hr. In non-stimulated cultures the incorporation of [^{14}C]thymidine was also measured during the first 24 hr of culture. DNA was extracted by trichloroacetic acid procedure and its radioactivity was measured in a Packard Liquid scintillation counter. The results are mean values of duplicate tubes.

Mitogens and antigens. PHA-W (batch No. K4944, Wellcome Reagent Ltd, Beckenham, Kent) was used at a concentration of 1 and 10 μg/ml. Concanavalin A (Con A, batch no. 4000, Pharmacia, Uppsala, Sweden) was used at the concentrations 20, 40 and 80 μg/ml. Pokeweed mitogen (PWM, batch no. 5211, Gibco, Berkeley, California) was used at the concentrations of 1 and 10 μg/ml. Lipopolysaccharide (LPS) from *E. coli* O55:B5 was prepared by phenol–water extraction (Professor T. Holme, Department of Bacteriology, Karolinska Institute, Stockholm, Sweden) and used at the concentrations of 10 and 100 μg/ml. Purified protein derivate of tuberculin (PPD, batch no. RP32, State Bacteriological Laboratories, Stockholm, Sweden) was used at the concentrations of 2·5 and 25 μg/ml.

Cytotoxic assay. Chicken red blood cells (ChRBC) labelled with [^{51}Cr]chromate (specific activity 50–150 mCi/mg chromium, Radiochemical Centre, Amersham, Bucks.) were used as target cells (Perlmann & Perlmann, 1971). 5×10^4 Labelled and washed target cells were mixed with $1{\cdot}25\times10^6$ lymphocytes (target cell:lymphocyte ratio 1:25) in complete Parker 199 medium with 5% heat-inactivated foetal bovine serum (FBS, Flow Laboratory, Ltd, Irvine, Scotland) and added to round-bottomed tissue culture tubes. Various dilutions (see Results section) of heat-inactivated (56°C, 1 hr) rabbit antiserum to target cell antigens was added. The final volume was 1·5 ml/culture tube. The tubes were incubated for 16 hr at 37°C in humidified air with 5% CO_2. Controls with target cells and lymphocytes ('spontaneous cytotoxicity') and controls with target cells without addition ('back-ground') were included.

Statistics. The mean values are given ± s.e. Student's *t*-test was used to estimate statistical significances of differences between mean values. Paired *t*-test analysis was used in the DNA synthesis experiments.

RESULTS

All the participants performed the ergometer work without difficulty. The haemoglobin and haematocrit rose during work (Table 1a). A marked rise in white blood cell counts was noted in all cases. Lymphocytes appeared to increase more than other leucocytes.

Lymphocyte subpopulations

The proportion of lymphocytes in the various subpopulations is shown in Table 1(b). The ergometer work resulted in a statistically significant increase of EAC-binding cells and of Ig-positive cells. A corresponding decrease of lymphocytes forming E rosettes was noted.

As no selective losses of lymphocytes occurred during the purification, the total number of lymphocytes in the subpopulations could be calculated (Holm *et al.*, 1975) (Table 1b). All three subpopulations increased. However, the increase of EAC-binding and Ig-positive cells by far exceeded that of E-binding cells. A total number of circulating B lymphocytes raised from >1/4 to about 1/3 of the total numbers of lymphocytes.

Double-staining cells

The sum of Ig-bearing or EAC-binding and E-binding cells was 90% and 91%, respectively. These figures were unchanged by work. When lymphocytes were stained for surface Ig and simultaneously

TABLE 1(a). Influence of physical activity on blood cells

	Haemoglobin (g/100 ml)	Haematocrit (%)	Total WBC (no./mm³)	Lymphocyte counts	
				Per cent	Total (no./mm³)
Before work	14·6±0·3	43·4±1·0	4879±411	34±2·3	1631±165
After work	16·5±0·3	49·0±0·9	8582±572	44±1·7	3783±252
n	12	12	14	15	14
P	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001

Mean no.±s.e.

TABLE 1(b). Influence of physical activity on lymphocyte subpopulations

	E-binding		Ig-bearing		EAC-binding		EA-binding	
	Per cent	Total no./mm³	Per cent	Total no./mm³	Per cent	Total no./mm³	Total	Total no./mm³
Before work	70±0·5	1148±120	20±0·3	329±34	22±0·5	356±38	21±0·9	366±66
After work	62±0·7	2325±166	29±0·8	1113±83	31±0·6	1185±84	26±2·3	1114±77
n	15	14	15	14	15	14	4	4
P	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001	< 0·05	< 0·001

Mean no.±s.e.

TABLE 2. Increase of double-reacting lymphocytes after work

	EAC+E		EAC+Ig+E		EA+E		EA+Ig+E	
	Before	After	Before	After	Before	After	Before	After
1	90	95	105	116	n.t.	n.t.	n.t.	n.t.
2	91	93	102	114	n.t.	n.t.	n.t.	n.t.
3	92	92	100	110	n.t.	n.t.	n.t.	n.t.
4	89	93	102	115	n.t.	n.t.	n.t.	n.t.
5	90	90	101	107	88	81	95	96
6	92	89	107	104	91	86	98	100
7	92	91	97	n.t.	93	90	97	n.t.
8	92	94	106	110	93	93	102	106
Mean±s.e.	91±0·5	92±0·8	103±1·3	111±1·8	91±1·4	88±3·0	98±1·7	100±3·6
n	8	8	8	7	4	4	4	3
P	n.s.		0·01		n.s.		n.s.	

n.s. = Not significant; n.t. = not tested.

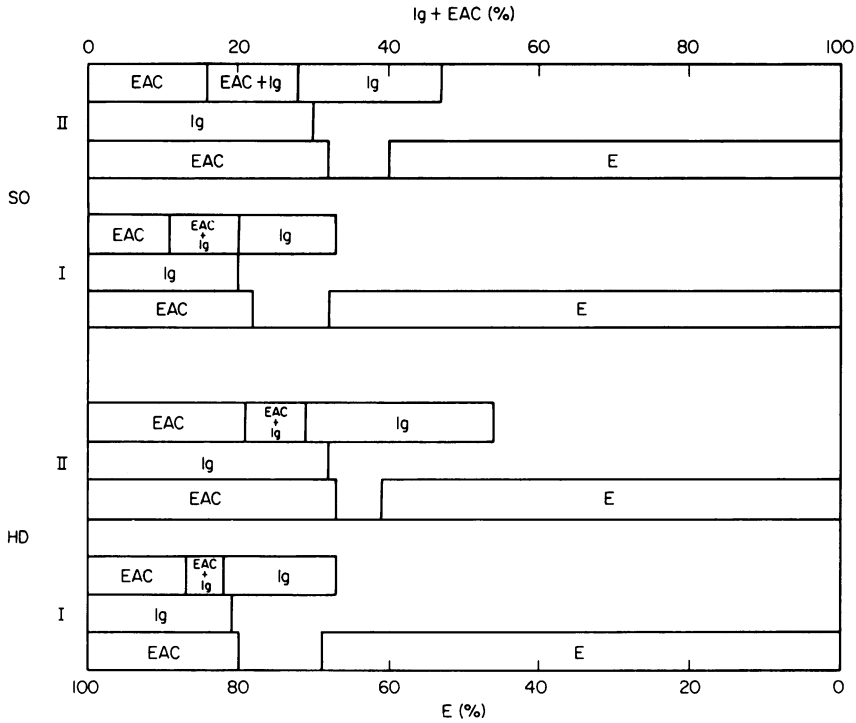

FIG. 1. Double-staining lymphocytes before work (I) and after work (II) in donors S.O. and H.D.

rosetted with EAC, three populations were detected (Table 2 and Fig. 1). One population reacted with both markers, the second carried Ig only, while the third only bound EAC. In lymphocytes collected at rest the sum of cells identified by these three markers and as E-binding cells was $103 \pm 1{\cdot}3\%$ (Table 2). In contrast, when lymphocytes collected after work were identified in the same manner, the sum was $111 \pm 1{\cdot}8\%$.

In both groups the total numbers of Ig-bearing or of EAC-binding lymphocytes identified by double staining were equal to those detected by individual staining with each marker (Table 2).

In two cases further investigated the addition of aggregated IgG (1 mg/ml) failed to inhibit rosettes formed with SRBC coated with IgG antibody and C. This concentration completely inhibits EA rosettes. This speaks against the presence of Fc receptors detected by the EA-rosetting technique on cells lacking the EAC receptor.

Double staining for surface Ig and Fc receptors was performed in four cases (Table 2). The sum of cells identified by this method and of cells forming E rosettes was about 100% and was not influenced by work.

Ig-bearing cells binding E were occasionally detected but never exceeded 2%. This was also true when lymphocytes were studied after work.

DNA synthesis

The thymidine incorporation by lymphocytes in the response to mitogens is shown in Table 3 and Fig. 2. Before work lymphocytes were stimulated by all mitogens. The response to LPS was weak, particularly at the lower dose. After work, thymidine incorporation into lymphocytes was significantly reduced in response to PPD, PHA, Con A and PWM. Stimulation of lymphocytes by LPS was not significantly altered.

A correlation was usually observed between the percentage of E rosettes and DNA synthesis induced by Con A in individual cases before and after work (Fig. 3).

TABLE 3. Influence of work on DNA synthesis in blood lymphocytes after activation with antigen and mitogens

	Incorporation of [^{14}C]thymidine (mean ct/min ± s.e.)			
	Before work	After work	*n*	*P*
Spontaneous, 24 hr	188 ± 21	222 ± 28	9	n.s.
Spontaneous, 3 days	199 ± 36	200 ± 30	9	n.s.
PPD (2·5 μg/ml)	4743 ± 1083	2886 ± 578	8	< 0·001
PPD (25 μg/ml)	4610 ± 1044	3072 ± 734	8	< 0·001
Con A (20 μg/ml)	13,247 ± 1097	10,123 ± 1639	7	< 0·001
Con A (40 μg/ml)	12,353 ± 975	9700 ± 1589	7	< 0·01
Con A (80 μg/ml)	8162 ± 1501	5022 ± 1365	7	< 0·01
PHA (1 μg/ml)	22,458 ± 859	20,591 ± 1097	9	< 0·01
PHA (10 μg/ml)	18,174 ± 842	15,320 ± 897	9	< 0·001
PWM (1 μg/ml)	9872 ± 867	7172 ± 766	9	< 0·001
PWM (100 μg/ml)	13,137 ± 1161	9283 ± 999	9	< 0·001
LPS (10 μg/ml)	1106 ± 297	848 ± 181	9	n.s.
LPS (100 μg/ml)	1864 ± 386	1439 ± 172	9	n.s.

n.s. = Not significant.

No differences were noted in the spontaneous DNA synthesis of lymphocytes before and after work whether measured after 24 or 72 hr of culture (Table 3).

Cytotoxicity

The isotope release induced by lymphocytes alone from chicken red blood cells was 3–10%. This did not exceed the background value. The cytotoxicity of lymphocytes against chicken red blood cells in the presence of antiserum was significantly increased after work (Fig. 4).

DISCUSSION

Physical exercise leads to leucocytosis which is not explained only by haemoconcentration (Tables 1a and 1b). Lymphocytes, particularly those of non-T origin, are more increased than other white blood

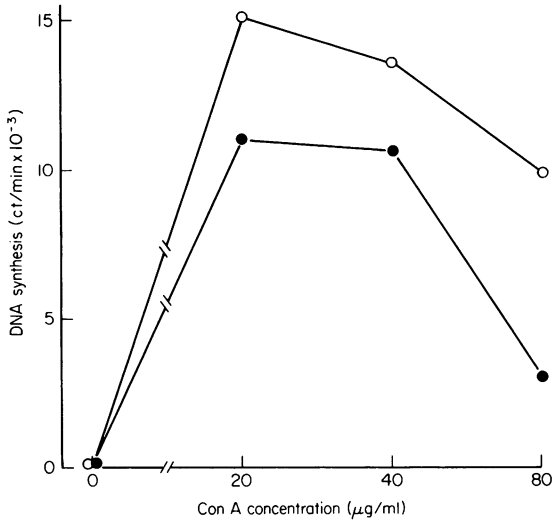

FIG. 2. Con A-induced DNA synthesis in lymphocytes harvested before (○) and after (●) work.

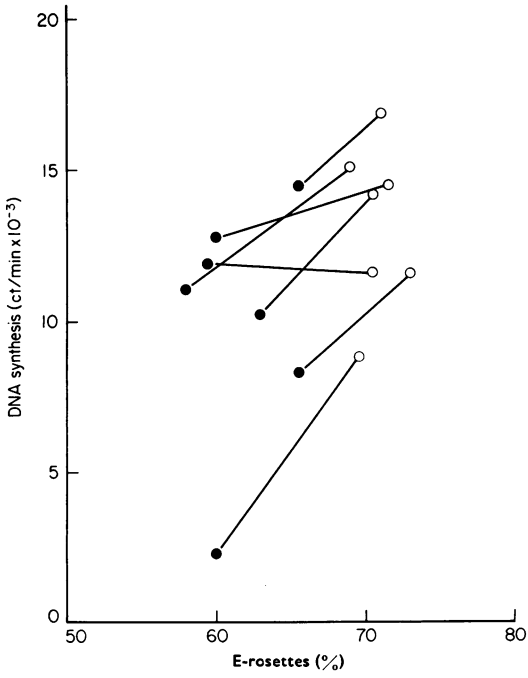

FIG. 3

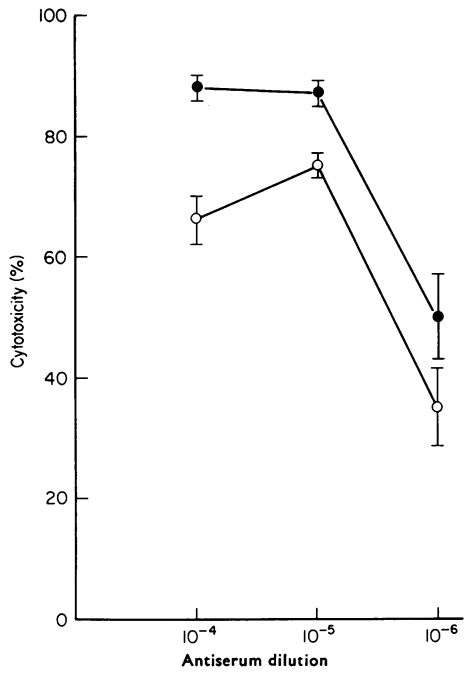

FIG. 4

FIG. 3. Correlation between E-rosetting cells and DNA synthesis induced by Con A, 20 μg/ml, in individual cases before (○) and after (●) work.

FIG. 4. Lymphocyte-mediated cytotoxicity against chicken RBC treated with rabbit antiserum before (○) and after (●) work. Mean %± s.e. $n=7$.

cells. The proportion of T and B lymphocytes varies between different lymphoid organs. Thus, the proportion of Ig-bearing cells is higher in the human spleen than in peripheral blood (Kaur *et al.*, 1974). The relative and absolute increase of cells with surface Ig noted after strenuous exercise may thus be compatible with their release from the spleen or other pools. However, B cells which are more sticky than other lymphocytes (Jondal, 1974a) might also be recruited during work from a pool sequestered by the laminar flow along the walls of the vessels. However, if this is true, a simultaneous granulocytosis should be expected after work. A selective decrease in disappearance and/or recirculation of B cells from the blood during work would also contribute to the B lymphocytosis. Since Ig-bearing cells identified by our method include a population of Fc-receptor carrying cells with absorbed IgG (unpublished data), increase of this population may also contribute to the high level of Ig-bearing cells noted after work.

Under basal conditions, the sum of E-binding and Ig-bearing or EAC-binding cells evaluated by our methods is rather constant with a mean value of 90·4% and 93·4%, respectively (Holm *et al.*, 1975). Ig-positive and/or EAC-binding cells identified by a double-staining technique and E-binding cells amounted to about 103% (Table 2). Under these conditions a small population of cells seems to react simultaneously with cell surface markers usually designed to B or T cells (Bentwich *et al.*, 1973; Dickler, Adkinson & Terry, 1974; Holm *et al.*, 1975). The double-reacting lymphocytes usually increased after work.

The finding of a large population of double-reacting lymphocytes after work may be interpreted in different ways. Some human blood lymphocytes binding SRBC may also react with B-lymphocyte markers (Dickler, Adkinson & Terry, 1974) but this double-reacting population is small, also after activation *in vitro* (Jondal, 1974b). The double-reacting cells may be constituents of a normally sequestered pool which is mobilized by work. Release of hormones, enzymes or other factors into the blood may also modify the cell surface thereby creating other binding properties (Bentwich *et al.*, 1973).

Activation of lymphocytes by antigens or mitogens is commonly used to characterize human lymphocyte subpopulations. Con A and PHA are known to activate T lymphocytes selectively (Chess, MacDermott & Schlossman, 1974). PWM activates both T and B lymphocytes (Douglas, 1972). However, the two cell types are stimulated by different constituents of PWM (Waxdal & Basham, 1974). The target cells for Con A, PHA and PWM may also be partially different T subpopulations (Stobo, Rosenthal & Paul, 1972). LPS which selectively stimulates B lymphocytes in mice also induced DNA synthesis and antibody production of human spleen cells (Andersson, Sjöberg & Möller, 1972; Möller, 1975). Activation of human peripheral blood lymphocytes is rarely possible unless very high doses (1 mg/ml) are used (Smith & Hammarström, personal communication).

Quantitative differences in the cell composition may determine varying DNA synthesis induced by mitogens and antigens before and after work. Thus, a close correlation was found between the DNA synthesis induced by Con A, PHA and PWM and the proportion of T lymphocytes (Fig. 3). Similarly, the decrease in proportion of T lymphocytes paralleled a decrease in DNA synthesis in response to PPD as expected from T cells being the target cell for PPD (Hedfors, 1974). However, the increased proportion of B cells after work did not result in augmentation of the DNA synthesis after activation with LPS or PWM under conditions which are assumed to stimulate B cells mainly (Mellstedt, 1975; Biberfeld & Mellstedt, 1974; Möller, 1975). This finding may suggest also qualitative alterations of the lymphocyte reactivity. The increase of non-T cells may also represent enlargement of subpopulations which are not reactive to the mitogens.

The cytotoxic activity clearly increased after work (Fig. 4). Different effector cells have been identified in the cytotoxic reaction with chicken red blood cells coated with IgG antibody. Thus, monocytes–macrophages as well as granulocytes may participate. Contamination of lymphocytes with such cells was not increased after work. Moreover, lysis of Chang cells, a human cell line, used as targets in some parallel experiments also increased after work. Such cells are insensitive to the lytic effect of granulocytes and monocytes (Huber & Holm, 1975; MacDonald & Bonnard, 1975). The lymphocytic effector cell in ADCC is defined as a non-T cell probably lacking surface Ig. The cells carry receptors for Fc and probably for C3. The origin of the cells is not known. Increase of cytotoxicity after work probably is caused by mobilization of effector cells which in this particular situation is associated with a rise of other non-T lymphocytes.

In conclusion, physical activity leads to increase and changes in the composition of peripheral blood lymphocytes. The sources of these cells and the mechanisms of their release deserve further studies.

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